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Papaya ringspot virus resistance of transgenic Rainbow and SunUp is affected by gene d sage, plant development, and coat protein homology

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Abstract

R1 plants of the transgenic papaya line 55-1, which expresses a single coat protein (CP) gene of the mild strain of the papaya ringspot virus (PRSV) HA from Hawaii, were previously shown to be resistant only to PRSV isolates from Hawaii. Two transgenic papaya cultivars were subsequently derived from line 55-1. UH SunUp (SunUp) is homozygous for the CP gene insertion and UH Rainbow (Rainbow) is hemizygous for the CP gene because it is a F1 hybrid of a cross between SunUp and the nontransgenic papaya cultivar Kapoho. To determine the various parameters that affect the resistance of SunUp and Rainbow, plants at different developmental stages (younger and older) were inoculated with PRSV isolates from Hawaii, Brazil, Jamaica, and Thailand. Hawaiian isolates shared nucleotide sequence identities of 96.7–99.8% to the CP transgene, and the other isolates shared sequence identities of 89.5–92.5%. Resistance was affected by CP gene dosage, plant developmental stage, and CP sequence identity of the challenge isolate. Young and older hemizygous Rainbow plants were resistant to the homologous PRSV HA (99.8% homology to CP transgene), while only older Rainbow plants were resistant to the other Hawaiian isolates (96.7% homology). However, all inoculated Rainbow plants were susceptible to PRSV isolates collected from Jamaica, Brazil, and Thailand. In contrast, SunUp was resistant to all PRSV isolates, except the one from Thailand, regardless of the plant developmental stage. Resistance to the Thailand isolate, which shares 89.5% homology to the transgene, was observed only with SunUp plants inoculated at an older stage. Steady state RNA analysis and nuclear run-on experiments suggested that resistance of the transgenic papaya is RNA-mediated via post-transcriptional gene silencing.

Introduction

The approach of pathogen-derived resistance has proved feasible for the control of various virus diseases (Sanford and Johnston, 1985). A number of laboratories have reported the successful establishment of virus resistance in transgenic plants expressing virus-derived genes or gene fragments (for review, see Lomonossoff, 1995). Moreover, high levels of stable resistance have been reported under natural virus infections and has led to the commercialization of transgenic virus resistant squash and papaya cultivars in the USA (Fuchs and Gonsalves, 1995; Gonsalves, 1998; Tricoli et al., 1995).

It is well established that the resistance can be either coat protein-mediated or RNA-mediated; and that RNA-based resistance is mechanistically similar to the phenomenon of gene silencing (Baulcombe, 1996). Typically, gene silencing occurs at transcriptional or post-transcriptional levels involving genes which contain regions of high similarity and is manifested by

reduced levels of steady state mRNA of the transgene and endogenous gene (Fagard and Vaucheret, 2000; Matzke and Matzke, 1998). Transcriptional gene silencing leads to a lack of transcription due to inactivation of the promoter, while in post-transcriptional gene silencing transcription occurs normally but RNA is degraded and thus lower steady levels are detected in the cytoplasm. Of the two kinds of gene silencing currently recognized, post-transcriptional gene silencing (de Carvalho et al., 1992; Finnegan and McElroy, 1994) has been linked to RNA-mediated transgenic resistance (Baulcombe, 1996). Additionally, the resistance is referred to as homology dependent post-transcriptional gene silencing or homology dependent resistance (Baulcombe, 1996) to reflect the specificity of the resistance mechanism.

Recently, Hawaiian solo papaya cultivars were transformed with a chimeric coat protein (CP) gene of PRSV HA 5-1, a mild mutant of PRSV HA from Hawaii (Fitch et al., 1992; Yeh and Gonsalves, 1984). Female R0 clones of the transgenic line 55-1 were resistant to a PRSV HA in the greenhouse (Fitch et al., 1992) and a field trial in Hawaii (Lius et al., 1997). R1 plants derived from a cross of a female R0 55-1 plant with nontransgenic Sunset, and thus hemizygous for the CP gene, were highly resistant to Hawaiian PRSV isolates HA (Yeh and Gonsalves, 1984) and Oahu (Tennant et al., 1994), but susceptible to PRSV isolates outside of Hawaii (Tennant et al., 1994). Two commercial cultivars UH SunUp (SunUp) and UH Rainbow (Rainbow) were subsequently derived from line 55-1 (Manshardt, 1998). SunUp is homozygous for the CP gene while Rainbow is hemizygous for the CP since it is a F1 hybrid of SunUp and the nontransgenic 'Kapoho' cultivar.

The long-term value of SunUp and Rainbow for controlling PRSV in Hawaii is dependent on their resistance to PRSV isolates in Hawaii and to potential isolates that may come into Hawaii. Thus, we have investigated the parameters that affect the resistance of SunUp and Rainbow to a range of PRSV isolates. Resistance against the isolates was found to be RNA-mediated and dependent on the CP transgene dosage, plant development stage, and sequence homology between the transgene and the isolates.

Materials and methods

Transgenic papaya lines. R0 and R1 generations of line 55-1 that express the CP gene of PRSV HA 5-1 were previously described by Fitch et al. (1992) and Tennant et al. (1994). SunUp is the R3 generation of line 55-1 and is homozygous for the CP gene. Rainbow is the F1 hybrid of a cross between SunUp and the nontransgenic 'Kapoho' papaya (Manshardt, 1998). To improve the rate of germination, seeds were incubated at room temperature in 1 M potassium nitrate (one volume of seeds to two volumes potassium nitrate) for 1 h (Nagao and Furutani, 1996) and then seeded in Cornell soil mix for germination. Seedlings were maintained under greenhouse conditions.

Southern assay. Southern assay was performed with DNA from transgenic Rainbow to estimate insertion copy number. DNA from seed-derived Rainbow grown in the greenhouse was isolated according to Cabrera (1995). Concentration and integrity of DNA were checked both spectrophotometrically (260 nm) and in agarose gels (Sambrook et al., 1989). DNA (15 µg) was digested with a 2-fold excess of *BglII*, *NcoI*, *EcoRI*, *ApaI*, *HindIII* or a combination of them at 37 °C for at least 18 h. Initial experiments showed that *BglII* does not cut the construct or the plasmid used to deliver CP and other transgenes to papaya plants (Fitch et al., 1992; Ling et al., 1991). Digested DNA was electrophoresed at 4 V/cm for about 16 h or until fragments of ca. 1 kb migrated to a position of about 7 cm from the meniscus of a 11-cm long gel. DNA was transferred to a positively charged nylon membrane using the alkaline method and subsequently fixed to the membrane by UV irradiation. The DNA was hybridized with purified PRSV CP DNA amplified by PCR from a plasmid with the CP gene (Ling et al., 1991) and labeled with ^{32}P-dATP (NEN Life Sciences Products) by random priming (Feingberg and Vogelstein, 1983). Pre-hybridization (2 h, 60 °C) and hybridization (24 h, 65 °C) steps were performed as recommended by manufacturer (NEN Life Sciences Products). After hybridization the membrane was sequentially washed once in 2×SSC for 5 min at room temperature, twice for 30 min in 2×SSC containing 1% SDS at 65 °C, once for 30 min in 0.5×SSC containing 1% SDS at 65 °C and in 0.1×SSC at room temperature for 10 min. The washed membrane was exposed to a radiographic film (X-Omat Blue XB-1, Kodak).

Analysis of transcript accumulation in transgenics. Total RNA was isolated from young leaves (top second or third leaf) of transgenic seedlings as described previously by Napoli et al. (1990). Ten µg of RNA were electrophoresed in a denaturing formaldehyde

1.2% agarose gel and blotting procedures were done according to the manufacturer's directions (DuPont Co., Boston, MA). Blots were probed with ^{32}P labeled *Nco*I fragment of the PRSV HA 5-1 CP gene (Feingberg and Vogelstein, 1983; Fitch et al., 1990; 1992; Quemada et al., 1990).

Isolation of nuclei and estimation of transcription rates. Isolation of nuclei and nuclear run-on transcription assays were done as originally described by Dehio and Schell (1994) and Pang et al. (1996). Similar amounts of *in vitro* labeled RNA transcripts were hybridized to identical Southern blot membranes containing 0.1 µg of coat protein gene (*Nco*I and *Hind*III PRSV HA 5-1 fragment), NPTII gene (*Bam*HI and *Hind*III fragment) and PCR product fragment of actin gene. Band intensities on photographed X-ray film were measured using the US NIH Image software (version 1.62).

Analysis of virus transgene expression in transgenics. Double antibody sandwich ELISA (DAS ELISA) was used to detect CP in plants using the protocol of Ling et al. (1991) and Tennant et al. (1994). A polyclonal antibody to the mild PRSV HA 5-1 from Hawaii was used to coat ELISA plates (2 mg/ml) and a monoclonal antibody to PRSV HA 5-1 conjugated to alkaline phosphatase (1 : 1500 dilution) was used for CP detection. Papaya leaf discs (2–7 mg from second leaf from the top) were homogenized in extraction buffer (1 : 50 w/v; 0.25 M potassium phosphate, 0.01 M ethylene diaminetetraacetic acid, disodium salt, pH 7.5). Duplicate samples were used in all assays. Two samples of papaya infected with PRSV HA 5-1 were included as positive controls and nontransgenic Sunrise papaya as negative controls. The reaction was read 1 h after the addition of substrate (1 mg/ml p-nitrophenyl phosphate) at 405 nm with a MicroELISA Autoreader (Dynatech Inc., Chantilly, VA).

Inoculation of plants with PRSV isolates. Transgenic and nontransgenic seedlings were mechanically inoculated with a 1 : 20 dilution (1 g leaf tissue to 20 ml of extraction buffer: 0.01 M potassium phosphate, pH 7.5) of leaf extracts of *Cucumis metuliferus* individually infected for 21 days with PRSV isolates from Hawaii, Brazil, Thailand, Jamaica (Tennant et al., 1994). The Hawaii isolates were designated as Hawaii (HA), Kapoho (KA), Oahu (OA), and Keaau (KE), while the isolates from outside of Hawaii were designated as Brazil (BR), Thailand (TH), and Jamaica (JA). These isolates were collected from infected papaya in the respective countries and maintained in the greenhouse in Geneva on papaya and *C. metuliferus* (Yeh and Gonsalves, 1984). Inoculum was applied to the three youngest fully expanded leaves of the plants predusted with carborundum (Universal Photonics, Inc., Hicksville, NY). All inoculated plants were observed daily for six weeks. Disease resistance was assessed by comparing the rate of symptom development (vein clearing, mosaic, leaf distortion, leaf reduction) and the severity of symptoms on new growth of transgenic and nontransgenic seedlings. Symptomless inoculated plants were reinoculated after 3 to 4 weeks.

Cloning and sequencing of the CP genes of severe PRSV isolates. Oligo primers designed to hybridize to a 17 bp region 27 nucleotides downstream from the PRSV translation stop codon and the CP cleavage sites described by Quemada et al. (1990) and Yeh et al. (1992) were used in reverse transcription polymerase chain reactions (RT-PCR) to generate the CP genes of the isolates KA, KE, JA, BR, and TH. The RT-PCR product of about 1 kb corresponding to the CP genes were digested with NcoI and ligated into the NcoI site of the plant expression vector pUC18CPexp (Slightom, 1991). Automated sequencing was done using a 373 Stretch DNA Sequencing System with Taq Dye terminator chemistry. All clones were sequenced in both directions using primers to the vector, the cloning primers, and primers designed at about 350 nucleotide intervals. Sequence data analysis was done with DNAStar biocomputing software (DNAStar Inc., Madison, WI).

Results

Homozygosity of a single CP gene insert switches the phenotype from susceptible to highly resistant to heterologous PRSV isolates

SunUp and Rainbow were derived from transgenic line 55-1 (Manshardt, 1998). SunUp is homozygous for the CP gene while Rainbow is hemizygous for the CP gene because it is a F1 hybrid from a cross of SunUp and the nontransgenic 'Kapoho'. Previously (Lius et al., 1997; Manshardt, 1998; Tennant et al., 1994), segregation patterns of the NPTII and GUS genes and of resistance had suggested that these transgenes were linked and segregated as a single genetic unit. Southern assay of DNA that was cut with single or combinations

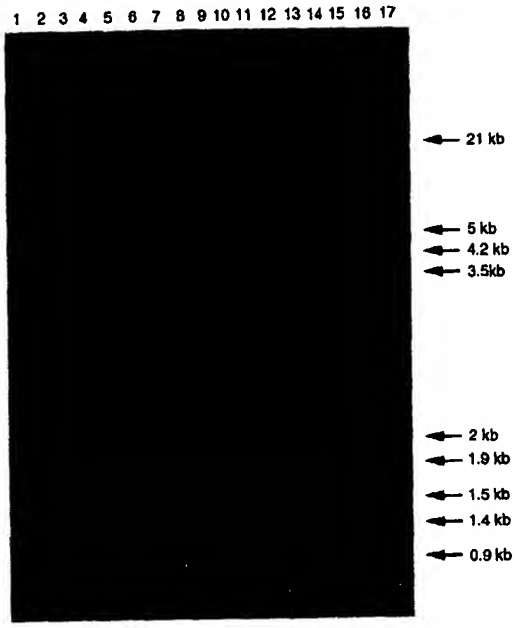

Figure 1. Southern assay of digested Rainbow genomic DNA. Fifteen μg of Rainbow DNA were digested with different combinations of restriction enzymes. The probe was a PCR product for PRSV HA 5-1 CP gene labeled with α-^{32}P-dATP. Lanes: 1, EcoRI; 2, ApaI; 3, HindIII; 4, NcoI; 5, BglII; 6, HindIII and NcoI; 7, HindIII and EcoRI; 8, HindIII and ApaI; 9, BglII and EcoRI; 10, BglII and ApaI; 11, NcoI and EcoRI; 12, NcoI and ApaI; 13, BglII, EcoRI and NcoI; 14, BglII, NcoI and ApaI; 15, EcoRI and ApaI; 16, HindIII and BglII; 17, NcoI and BglII.

of enzymes showed that Rainbow contained only one insert of the CP gene (Figure 1). Unequivocal evidence for Rainbow having only one insert was obtained with the use of *BglII* enzyme that does not cut the plasmid harboring the transgene construct in combination with other enzymes that cut the plasmid once (*ApaI, EcoRI*), twice (*HindIII*), or with an enzyme (*NcoI*) that excises the CP gene (Figure 1).

Previous results (Tennant et al., 1994) had shown that R1 plants of line 55-1 were resistant to the homologous PRSV HA but susceptible to PRSV isolates outside of Hawaii. These plants were from a cross of a female R0 plant of line 55-1 with a nontransgenic 'Sunset', resulting in transgenic progenies that were hemizygous for the CP gene of PRSV HA 5-1. Thus, we were interested in similarly testing SunUp and Rainbow (Manshardt, 1998). SunUp and Rainbow were challenged with the homologous PRSV HA, three heterologous isolates from Hawaii (OA, KA, and KE) and three isolates originating outside of Hawaii (BR, TH, JA) (Tennant et al., 1994).

In experiments performed in January–March with seedlings about 6–9 cm tall, we were surprised to observe that Rainbow exhibited high levels of resistance only to the homologous HA isolate and were susceptible to the heterologous Hawaiian isolates OA, KE and KA with 86–100% infection (Table 1). However, the timing of symptom development and severity of symptoms were different from those observed on nontransgenic plants. Rainbow seedlings showed symptoms one to seven weeks after similarly inoculated nontransgenic plants. After initial symptom expression, there was a period (2–28 days) where the

Table 1. Reactions of Rainbow, and SunUp papaya to inoculations with PRSV isolates during January–March and July–March periods

Test plants[a]	Resistance (%) to PRSV isolates, experiment period, plant height[b]								
	Jan–Mar	Hawaii				Jul–Mar	Outside Hawaii		
		HA	OA	KA	KE		JA	BR	TH
Rainbow (cp/+)	6–9 cm	86	14	0	0	6–20 cm	0	0	0
SunUp (cp/cp)	3–20 cm	100	100	100	94	3–20 cm	100	100	0
Sunrise (+/+)	6–9 cm	0	0	0	0	6–20 cm	0	0	0

[a]Rainbow is hemizygous (cp/+) and SunUp homozygous (cp/cp) for the PRSV CP transgene. Derivation of Rainbow and SunUp is described in Materials and methods. Sunrise (+/+) is nontransgenic.
[b]Isolates HA and OA are from Oahu island, KA and KE from Hawaii island, JA = Jamaica, BR = Brazil, TH = Thailand. At least 10 plants were inoculated with each isolate in 2–5 experiments. Rainbow, and Sunrise plants were 6–15 weeks old at time of inoculation, and SunUp plants were 3–16 weeks old. All SunUp inoculations were done in Jan–Mar period. Plants grew slowly in Jan–Mar period. Resistant plants were inoculated twice. Plants were observed for 56 days after inoculation.

new leaves of the infected plants did not exhibit the symptoms. However, symptoms then reappeared but in a milder form.

Rainbow was also susceptible to heterologous isolates outside of Hawaii (Table 1). Unlike the above observations from inoculation with Hawaiian isolates, however, transgenic seedlings developed severe symptoms that did not become milder on newer developing leaves. A delay of 1–38 days in symptom development was observed. The experiments were done over the summer and winter months with seedlings 6–20 cm tall.

In contrast, the homozygous SunUp was resistant against all tested PRSV isolates except that of Thailand (Table 1). These experiments were done during the winter months with plants that were inoculated at three weeks after germination (3–6 cm, younger than usual), six weeks (6–15 cm), and 15 weeks (9–21 cm). Moreover, the resistance held up to two mechanical inoculations and did not break down during the two months plants were maintained in the greenhouse. Although SunUp at these ages did not afford resistance to the PRSV isolate from Thailand, symptoms did not appear on inoculated plants until a long delay of four to six weeks. Apart from the difference in the rate of infection there was no observable difference in the severity of symptoms on SunUp plants and similarly inoculated nontransgenic plants. Plants inoculated at 3–15 weeks responded similarly.

Plant developmental stage influences level of resistance

An observation made in the greenhouse prompted further investigations into whether Rainbow and SunUp would become more resistant to PRSV at later stages of plant development. In two greenhouse inoculation studies, five inoculated R1 55-1 plants were susceptible when inoculated at 6 weeks after germination but plants from the same germination batch were resistant when inoculated at 16 weeks after germination.

Thus, inoculation experiments with the four isolates from Hawaii were conducted with Rainbow seedlings at 14 and 17 weeks (Table 2) between the months April and June. Seedlings that were at 14 weeks of age with an average height of 13 cm were resistant to the homologous HA isolate, partially resistant to OA and KA isolates (33–62%) and susceptible to KE. However, at 17 weeks and an average height of 46 cm, seedlings were resistant to all four isolates. The plants remained symptomless following two inoculations and for a period of three months in the greenhouse after the final inoculation.

Table 2. Influence of plant development on resistance of transgenic Rainbow and SunUp to PRSV isolates from Hawaii and Thailand

Cultivars[a]	Characteristics of plants[b]		Resistance (%) to PRSV isolates[c]				
	Age (weeks)	Height (cm)	HA	OA	KA	KE	TH
Rainbow (cp/+)	14	13 (6-20)	100	62	33	0	
	17	46 (34-60)	100	100	100	100	
SunUp (cp/cp)	16	15 (15)					0
	23	38 (17-59)					75
	29	93 (70-117)					100
Sunrise (+/+)	14–29	50 (24-69)	0	0	0	0	0

[a]Rainbow and SunUp are hemizygous (cp/+) and homozygous (cp/cp) for CP gene, respectively. Sunrise is nontransgenic (+/+) papaya.

[b]Plants were inoculated at indicated ages (in weeks) and height (average with range in parenthesis).

[c]Isolates HA and OA are from Oahu island, KA and KE from Hawaii island, and TH from Thailand. At least 15–20 plants were inoculated with each isolate over three experiments, except that 8 plants of 23-week-old SunUp were inoculated with PRSV TH. Experiments were done in April–June. Plants were observed for 56 days after inoculation.

Similar inoculation experiments with the TH isolate to SunUp were done between April and June. The seedlings were inoculated at 16 (average height 15 cm), 23 (average height 38 cm), and 29 (average height 93 cm) weeks after germination. Sixteen-week-old plants were not resistant to the virus but resistance was obtained with 23- and 29-week-old plants (Table 2).

Taken together, our results clearly showed that the resistance to the transgenic plants increased as the plants got older. The CP hemizygous line 55-1 and Rainbow were susceptible to PRSV isolates from Hawaii at a younger stage but resistant when inoculated at an older stage. Furthermore, the CP homozygous SunUp was susceptible to the TH isolate when inoculated at a younger stage but resistant at older stages.

Evidence that resistance is due to post-transcriptional gene silencing

The CP levels in transgenic plants were routinely determined for test plants by ELISA. Data from 306 tested SunUp and Rainbow plants showed that SunUp had consistently lower levels of CP than Rainbow (Table 3). For example, 80% of the SunUp plants had OD 405 nm ELISA readings below 0.059 compared to 26% for Rainbow, while only 6% of the SunUp plants had readings of 0.1–0.5 compared to 33% for Rainbow.

Table 3. Relative expression of PRSV CP in transgenic Rainbow and SunUp that are hemizygous and homozygous for CP transgene, respectively

	No. of plants (% of total no. plants)	
OD 405 nm	Rainbow	SunUp
0.00–0.05	39 (26)	125 (80)
0.06–0.09	62 (41)	22 (14)
0.10–0.5	49 (33)	9 (6)
Total	140 (100)	156 (100)

OD 405 nm is absorbance in ELISA tests one hour after substrate incubation. Nontransgenic papaya averaged readings of 0.00.

Since the average CP expression levels of the CP homozygous SunUp was much lower than the CP hemizygous Rainbow, nuclear run-on reactions were done to determine whether the lower levels were due to decreased transcription or post-transcriptional gene silencing. Nuclei were isolated from SunUp, Rainbow, R1 55-1, and nontransgenic Sunrise papaya. Four experiments were done with material from different nuclei isolations. Figure 2 shows a typical result of a nuclear run-on experiment. There was a clear and consistent difference in signal intensity between homozygous SunUp and hemizygous R1 55-1 and Rainbow lines: higher transcription in homozygous than in the hemizygous plants. This was in contrast to the steady state levels of the transgene transcript in Northern assays (Figure 3) where SunUp had lower or equal levels of CP transcripts than Rainbow. These results clearly suggested that reduced steady state transgene RNA was due to post-transcriptional transgene silencing.

Figure 2. Comparative nuclear run-on transcription assays of nuclei isolated from leaves of CP hemizygous 55-1 and Rainbow and CP homozygous SunUp transgenic papaya. Nuclear transcripts of Rainbow 36 and SunUp 428 plants were hybridized with ^{32}P labeled probes specific to Actin (A), PRSV HA 5-1 coat protein (CP), or NPTII (N) genes. The CP/A ratio of labeled transcript for each run-on was 1.7, 2.0, and 3.8 for 55-1, Rainbow 36, and SunUp 428, respectively. Note that northern blots were also done for Rainbow 36 and SunUp 428 plants as shown in Figure 3.

Resistance and degree of homology of CP genes of PRSV isolates with transgene

Given the observation that the transgenic papaya had limited protection against PRSV isolates from outside of Hawaii, it was of interest to compare the sequences of the CP genes of these isolates to that of the PRSV HA 5-1 CP transgene. Isolates selected for analysis included PRSV HA, OA, KA, KE, JA, BR, and TH. Nucleotide identities of the isolates were compared with the published sequence of PRSV HA 5-1 (Quemada et al., 1990).

Nucleotide identities between the CP sequences of the isolates and PRSV HA 5-1 ranged from 89.5% to 99.8% (Table 4). Isolates from Hawaii had the highest sequence identities with PRSV HA 5-1 (96.7–99.8%). Interestingly, of the Hawaii isolates, the KE and KA isolates from Hawaii island had the lowest sequence homology to the transgene and also induced the highest percentage infection on hemizygous plants inoculated at a young plant stage (Tables 1 and 2). The most

distantly related isolate to the transgene was PRSV TH, which also caused the most infection to all transgenic papaya plants (Tables 1 and 2).

As expected, the core region of the CP gene was the most conserved between isolates with percent similarities ranging from 97% to 99% between the Hawaiian isolates and PRSV HA 5-1, and 90–95% between the PRSV HA 5-1 and isolates outside of Hawaii. Similarly, the CP carboxy terminus was highly conserved among the isolates (91–100%) (Table 4). The most variable region among the isolates was the N terminus region. Percent similarities between the N terminal region among isolates outside of Hawaii and PRSV HA 5-1 were 83.7%, 84.4%, and 89.3% for TH, BR, and JA, respectively. In contrast, higher percent similarities of 95.3–99.3% were observed with Hawaii isolates for the N terminal region.

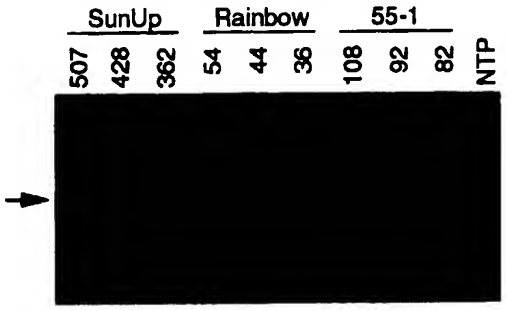

Figure 3. Northern blot assay of PRSV CP transgene from CP hemizygous 55-1 and Rainbow and CP homozygous SunUp transgenic plants. Total RNA (10 μg) were loaded in each well and separated by electrophoresis in an agarose gel under denaturing conditions. A ^{32}P labeled DNA probe specific for the PRV HA 5-1 coat protein sequence was used to detect the CP transgene. Arrow denotes the position of the transgene. 507, 428, 362; 54, 44, 36; and 108, 92, and 82 represent three different plants of each of SunUp, Rainbow, and 55-1, respectively while NTP is RNA from a nontransgenic plant.

Discussion

We have shown that resistance of the commercial transgenic papaya SunUp and Rainbow cultivars to a range of PRSV isolates is dependent on gene dosage, plant age and is RNA-mediated via post-transcriptional gene silencing. Furthermore, our evidence suggests that the resistance is also affected by the sequence homology that the attacking PRSV isolate has to the CP transgene.

Previous reports have shown that RNA-mediated resistance is affected by plant development stage, gene dosage, and homology of the attacking virus with the transgene. However, these have primarily dealt with different viruses, and not with a range of isolates of a virus, and have not dealt with transgenic plants that are being grown in the field under commercial conditions. For example, Goodwin et al. (1996) showed a correlation between level of resistance to TEV and number of nontranslatable coat protein transgenes in transgenic plants, and Pang et al. (1996) also showed the effect of gene dosage and plant developmental

Table 4. Summarized reactions and coat protein nucleotide sequence homologies of PRSV isolates inoculated to Rainbow and SunUp transgenic papaya expressing coat protein transgene of PRSV HA 5-1

PRSV isolates	% Homology of coat protein[a]				Overall	Reaction[b] of isolates	
	N	Core	*C*	3′ncr		cp/+	cp/cp
Hawaii-HA	99.3	99.8	100	100	99.8	R	R
Hawaii-OA	97.3	98.0	100	95.7	97.9	sR	R
Hawaii-KA	95.3	97.1	98.3	93.6	96.7	sR	R
Hawaii-KE	95.3	97.1	98.3	93.6	96.7	sR	R
Jamaica-JA	89.3	95.0	91.5	69.6	92.5	S	R
Brazil-BR	84.4	93.9	98.3	73.3	91.6	S	R
Thailand-TH	83.7	90.7	91.5	89.4	89.5	S	sR

[a]Nucleotide sequences of PRSV HA 5-1 from Quemada et al. (1990) and PRSV HA from Yeh et al. (1992). All other sequences from Gonsalves laboratory. The N = 119 nt of N terminus, core = 641 nt of the core, C = 59 nt of carboxy terminus, and 3′ncr = 35 nt of non-coding region following the PRSV CP stop signal.

[b]R = resistant, sR = some plants susceptible when inoculated at young stage, while plants are resistant at older stage. See Tables 1 and 2. cp/+ = Rainbow which is hemizygous for cp, and cp/cp = SunUp which is homozygous for cp.

stage with tomato spotted wilt tospovirus, but different isolates of the virus were not tested. Two recent reports suggest that gene dosage can affect resistance of a transgenic line to virus isolates. McDonald et al. (1997) reported that homozygous plants of a transgenic tobacco line with the transgene of potato virus Y strain N (PVY^N) were resistant to strains of PVY, PVY^N, and PVY-36, whereas hemizygous plants were less resistant. In lettuce mosaic virus transgenics, Dinant et al. (1997) reported that homozygous, but not hemizygous, R2 progeny were able to confer a level of resistance to lettuce mosaic virus isolates LMV-1, LMV-E, and LMV-13.

The hemizygous yellow-flesh Rainbow is favored by farmers over SunUp and is now widely planted on Hawaii Island to replace the nontransgenic Kapoho. As shown in this report, however, a percentage of young Rainbow seedlings can be infected by PRSV isolates from Hawaii in the greenhouse and recent field experiments in which plants are mechanically inoculated at younger or older stages have given similar results (Ferreira, personal communication). Thus, the practical benefits derived from the resistance of Rainbow may be compromised if numerous seedlings become infected before reaching the resistance stage, or if various isolates already in Hawaii are able to overcome the resistance of Rainbow at any stage of development. Extensive observations of large fields of Rainbow growing next to severely infected Kapoho show no breakdown of resistance in Rainbow, indicating that the susceptibility of Rainbow at a very young stage has not had any practical impact. Furthermore, a large collection of PRSV isolates from different locations in Hawaii failed to infect older Rainbow plants (Ferreira, personal communication). Nevertheless, monitoring Hawaii for PRSV isolates that may overcome the practical resistance of Rainbow needs to be done on a continuing basis.

Since resistance via post-transcriptional gene silencing is sequence homology-dependent, it is logical to surmise that the lack of resistance of Rainbow to isolates of PRSV outside of Hawaii is due to their lower sequence homology to the CP transgene. Recent evidence from our laboratory provide experimental evidence that this is the case. Although PRSV HA does not overcome the resistance of Rainbow or SunUp (Tables 1 and 2), a recombinant virus of PRSV HA in which the CP gene was replaced with the CP gene of PRSV YK (Wang and Yeh, 1997), which has only 88.5% homology to the CP transgene, was able to infect Rainbow and SunUp (Chiang and Gonsalves, unpublished results).

In summary, we have shown that the development of transgenic plants with resistance against viral populations is complicated by factors such as transgene dosage, plant developmental stage, sequence identity between transgene and virus isolate populations. From the practical standpoint, the degree of resistance can be increased by increasing transgene dosage and by using transgenes with segments (Pang et al., 1997) that have sequence homology to prevalent virus isolates. In fact, we recently showed that a single chimeric transgene consisting of the CP gene of turnip mosaic virus linked to a 216 bp segment of the N gene of tomato spotted wilt virus conferred resistance to both viruses in *Nicotiana benthamiana* (Jan et al., 2000). Such approaches that combine gene dosage with transgenes that confer multiple resistance to viral strains are valuable since the transgenic plants will be exposed to virus populations and varied growth conditions in the field.

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